# Validation of Antibodies for the Immunolocalization of Germ Cells in Alligator Gonads.

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## Introduction

Previous studies of alligators from pesticide-contaminated lakes have shown decreased fertility in comparison to less polluted areas (1). Our research is focused on elucidating mechanisms of decreased reproductive success in alligators exposed to environmental contaminants during embryonic development. Localization and identification of germ cells can provide critical information in assessing future reproductive capability. Germ cells are the unique precursors of gametes, more commonly referred to as eggs and sperm.

Specific cell types, including germ cells, can be identified through immunohistochemistry (IHC), which relies on antibodies that bind specific molecules (antigens) present in the cells of interest. Primary antibodies are intended to recognize and bind the relevant antigen. Direct detection involves primary antibodies that have been conjugated to a fluorophore or enzyme for detection. Indirect detection, a more commonly used method, involves the addition of a conjugated secondary antibody, which amplifies the signal relative to direct detection (Fig. 1).

In this study we attempted to validate two antibodies for use as IHC markers of hatchling alligator germ cells. We tested a rabbit polyclonal antibody to mouse DDX4, an RNA helicase produced in germ cells, and a mouse monoclonal antibody to hamster SCP3, a marker of prophase I.

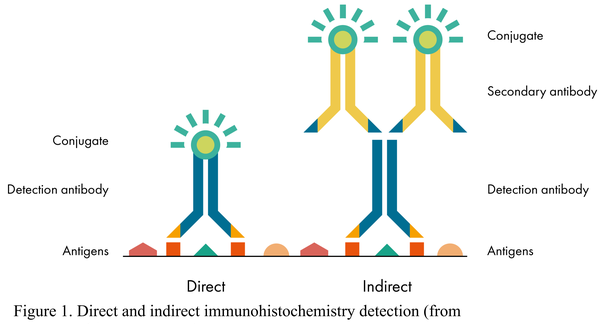


Figure 1. Direct and indirect immunohistochemistry detection (from www.azurebiosystems.com).

## Objectives

1. Validate immunohistochemistry technique using mouse as a positive control.
2. Immunolocalize germ cells in hatchling alligator gonads.
3. Comparison of results with enzyme conjugated and fluorophore-conjugated secondary antibody.

## Methods

### 1. Tissue Processing

Hatchling alligator gonad-adrenal-mesonephric kidney (GAM) complexes were formalin fixed, dehydrated, cleared, and imbedded in paraffin wax. GAM complexes were sagittally sectioned at 5 µm and mounted on positively charged slides.

### 2. Immunohistochemistry: Antigen Retrieval and Blocking

Slides were deparaffinized and rehydrated prior to antigen retrieval, during which slides were immersed in citrate buffer (pH 6.0) under pressure at 110ºC for 20 minutes. Blocking (Fig. 2) was performed by incubating sections in Tris-buffered saline (TBS) containing 0.25% Triton X-100, 1% bovine serum albumin (BSA), and 10% normal goat serum (NGS) for 1 hour at room temperature.

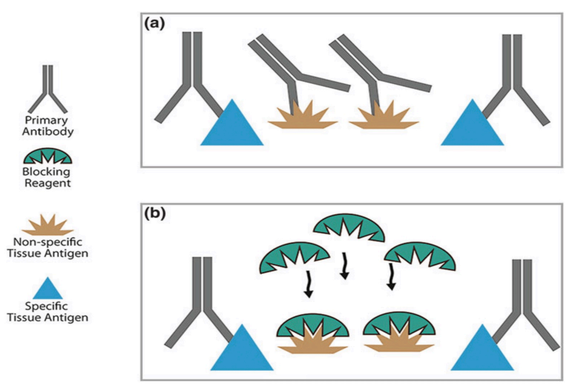


Figure 2. Immunohistochemistry blocking step (2).

### 3. Immunohistochemistry: Primary and Secondary Antibodies

Primary antibody was diluted in TBS with 0.02% Triton X-100, 1% BSA, and 10% NGS prior to overnight incubation at 4ºC.

Prior to incubation with secondary antibodies conjugated with horseradish peroxidase (HRP), tissue sections were treated with 0.3% $H_2O_2$ to reduce endogenous peroxidase activity. Following a 1-hour incubation with HRP-conjugated antibody, slides were developed with the addition of 3,3′-diaminobenzidine (DAB). Hematoxylin counterstaining was performed prior to coverslipping.

Slides incubated with secondary antibodies conjugated with Alexa Fluor 488 were coverslipped with VectaShield mounting medium and allowed to cure for 48 hours prior to sealing.

## Results

Positive control mouse testes stained with DDX4 primary and HRP conjugated secondary antibodies showed staining specific to germ cells. Staining of alligator GAM appeared specific to putative germ cells in the ovarian cortex and seminiferous tubules(Fig. 3).

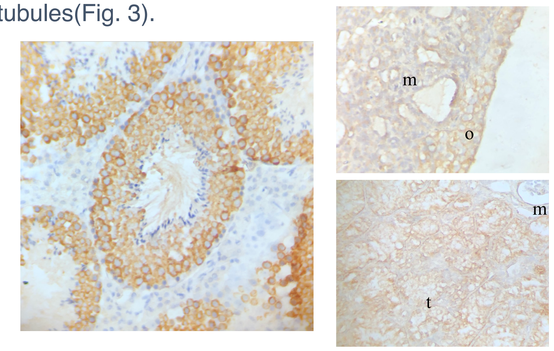


Figure 3. Mouse testis (left), female alligator GAM (top-right), and male alligator GAM (bottom-right) stained with anti-DDX4 (Abcam 13840 at 1:150) and counterstained with hematoxylin. Abbreviations: m, mesonephric kidney; o, ovary; t, testis.

Detection with fluorophore-conjugated secondary antibody in mouse testes was specific to cytoplasmic staining of spermatogonia and spermatocytes. Staining in alligator GAMs was not specific to putative germ cells in the ovarian cortex or seminiferous tubules (Fig. 4).

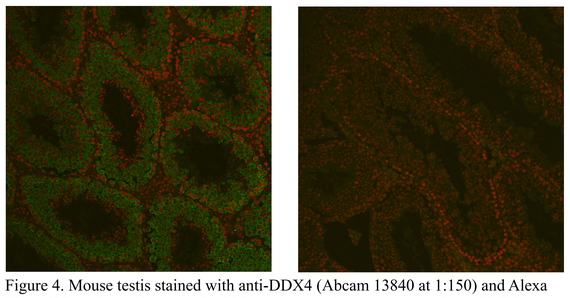

Figure 4. Mouse testis stained with anti-DDX4 (Abcam 13840 at 1:150) and Alexa Fluor 488 conjugated goat anti-rabbit (green), nuclear counterstaining with TO-PRO-3 (red), (left), and mouse testis with no 1º antibody, (right).

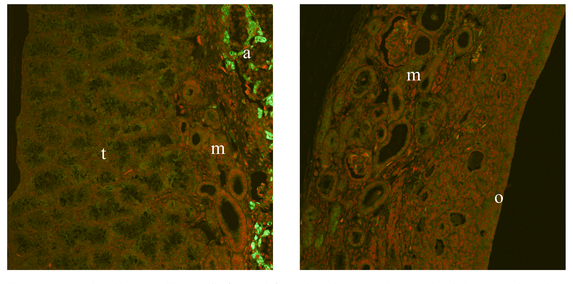


Figure 5. Male alligator GAM (left) and female alligators GAM (right) stained with with anti-DDX4 (Abcam 13840 at 1:150) and Alexa Fluor 488 conjugated goat anti-rabbit (green), nuclear counterstaining with TO-PRO-3 (red). Abbreviations: a, adrenal gland; m, mesonephric kidney; o, ovary; t, testis.

## Conclusions

The IHC protocol used for the immunolocalization of germ cells expressing SCP3 and DDX4 worked as expected in the positive control mouse tissue. Our initial results using HRP-conjugated secondary antibodies suggested that the monoclonal antibody to SCP3 did not recognize homologous antigens in alligator (results not shown), whereas staining with the polyclonal antibody DDX4 was most intense in the region of putative germ cells in neonatal alligator gonads. Further testing with fluorophore-conjugated secondary antibody contradicted those results, with high background staining throughout the GAM, and the most intense staining in adrenal tissue. Background staining in sections incubated with secondary antibody only was negligible, suggesting that our primary antibody is responsible for most of the non-specific binding. We believe the discrepancy is due to the lower resolution offered with the HRP-DAB system.

Our results underscore the challenges of IHC, particularly in species for which antibodies are not readily available. The ability to immunolocalize germ cells in alligators will aid in our understanding of the effects of environmental contaminant exposure; therefore, we will continue test candidate antibodies for this purpose.

## References

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